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(54) Title: CYCLOSPORINS TO TREAT ALZHEIMER'S DISEASE

(57) Abstract: Non-immunosuppressive, cyclophilin-binding cyclosporins, are useful as neuroprotective agents, e.g. in the prevention or treatment of pathological conditions associated with AB secretion and/or production.

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CYCLOSPORINS TO TREAT ALZHEIMER'S DISEASE

The present invention relates to novel uses of cyclosporins, and in particular to new pharmaceutical uses of non-immunosuppressive, cyclophilin binding cyclosporins.

Cyclosporin A (CsA) binds to immunophilin proteins such as cyclophilins (CyP), whereas FK506 and rapamycin, both immunophilin-binding compounds bind to FK506-binding proteins (FKBP). Although immunophilin-binding is required, it is not sufficient for the immunosuppressive activity of these drugs. Biological effects are observed upon interaction of the drug/immunophilin complexes with a third effector protein. For example, the CyP-CsA and FKBP-FK506 complexes inhibit the serine/threonine phosphatase activity of calcineurin, thereby blocking the production of cytokines such as interleukin-2. On the other hand, the FKBP-rapamycin complex inhibits a kinase called FRAP (also known as RAFT or mTOR) 5 that is involved in interleukin-2 receptor-mediated T cell proliferation.

A new class of compounds named sanglifehrins, have been isolated from *Streptomyces* sp. A92-308110. Among the 20 different sanglifehrins isolated so far, sanglifehrin A (SfA) is the most abundant compound and displays potent immunosuppressive activity. SfA represents a novel type of immunosuppressant whose mode of action is different from that of all other known immunophilin-binding compounds, namely CsA, FK506, and rapamycin. SfA has been shown to bind directly to the immunophilin protein, cyclophilin D (CyD), at a distinct site from another immunophilin, cyclophilin A (CpA), at the mitochondrial transition pore (MTP) complex and inhibit MTP opening.

Non-immunosuppressive, cyclophilin binding cyclosporins and their use in the treatment and prevention of AIDS and AIDS-related disorders are described in European Patent no. 484281, which includes a general description of the cyclosporin class of compounds, their nomenclature and mode of action. The disclosure of EP 0,484,281 B, in particular the general description referred to above and other parts of the description referred to hereinafter, is included by reference in the teaching of the present application.

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Surprisingly, it has now been found that cyclosporins which bind to cyclophilin, but are not immunosuppressive, are useful as neuroprotective agents to treat pathological conditions associated with A β production and/or secretion, including but not limited to Alzheimer's Disease ('AD'). AD is characterized by the extracellular accumulation of amyloid plaques in the brain composed primarily of the A β 40 or 42 amino acid peptides. The extracellular accumulation of these peptides is a hallmark pathology of the disease (Selkoe 1999). The A β peptide is generated by the endoproteolytic cleavage of the amyloid precursor protein (APP), a ubiquitously expressed type I transmembrane protein (Selkoe 1999; Sisodia 2000). The two enzymes that cleave APP in the amylogenic pathway are called the β - and γ -secretases, which cleave APP from the N- and C-termini, respectively. In this pathway, the β -secretase (BACE1) is the first enzyme to cleave APP, producing a secreted sAPP β fragment and a membrane associated C-terminal fragment (CTF, C99) (Vassar, Bennett et al. 1999). The C99 fragment is the substrate for the γ -secretase complex (GACE) which cleaves C99 to produce A β and AICD (APP IntraCellular Domain). AICD binds a complex with Tip60 and Fe65 which derepresses KAI1 (a tetraspanin cell surface molecule), a gene in the NF κ -B pathway (Baek, Ohgi et al. 2002). The GACE complex is made up of four primary components, presenilin 1 (PS1), nicastrin (NCSTN), Aph1, and Pen2 (Edbauer, Winkler et al. 2003; Kimberly, LaVoie et al. 2003). The PS1 functional homologue, presenilin 2 (PS2) contributes to ~20% of the A β produced in a cell (Kimberly, Xia et al. 2000). Although these four components are necessary and sufficient to reconstitute GACE activity, there is evidence of non-GACE mediated cleavage of APP resulting in A β production (Tesco, Koh et al. 2003), (Nunan, Shearman et al. 2001).

DETAILED DESCRIPTION OF THE INVENTION

The elucidation of novel genes involved in the APP pathway is a critical step in determining complete etiology of the disease as well as developing a better understanding of the complex mechanism driving A β production. The determination of new genes and pathways regulating A β aids in developing novel therapeutic strategies to treat the progression of the disease. Several genetic links and chromosomal regions have been specifically associated with Late Onset Alzheimer's Disease (LOAD) (>65 years of onset) (Ertekin-Taner, Graff-Radford et al. 2000), (Bertram, Blacker et al. 2000), (Scott, Hauser et al. 2003). A subset of LOAD associated genes, as well as novel genes involved in APP processing are described in copending USSN __, which includes a description of large-scale

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functional screen to test cDNA clones for their ability to modulate A β production in CHO K1 cells. The disclosure of USSN ___, in particular the identification of genes which modify AB secretion and other parts of the description referred to hereinafter, are included by reference in the teaching of the present application.

The discovery of genes found in the immunophilin pathway which serve as critical regulators of A β production in cells provides suitable drug targets in Alzheimer's disease . Some of the cDNAs discovered in the functional screen which are implicated in the immunophilin pathway include Map kinase 4, Calmodulin, acid ceramidase, and TOB3, a AAA-ATPase.

Mitogen-activated protein kinases (MAPKs), also known as extracellular signal-regulated kinases (ERKs), are a family of serine/threonine protein kinases that phosphorylate and positively or negatively regulate target substrates initiating signaling cascade events. ERKs transduce signals from the cell membrane to the nucleus in response to a wide range of stimuli and play an important role in modulating gene expression, mitosis, proliferation, motility, metabolism, and apoptosis (Wada and Penninger 2004). Inhibitors of MEK and ERK activity are shown to inhibit APP catabolism. In addition, MAPK can activate JNK and trigger apoptosis, a process known to increase A β production (Tesco, Koh et al. 2003). Although not wishing to be bound by theory, the MAPK4 cDNA could be acting through APP catabolism or activation of apoptosis.

As well, the APP intracellular domain (AICD) fragment interacts with cJun N-terminal kinase (JNK) linking the MAP kinase pathway to APP processing (Scheinfeld, Roncarati et al. 2002). MAPK4 is an ERK that activates JNK and therefore the overexpression of MAPK4 could lead to hyperactivation of JNK which could increase JNK-AICD interactions leading to increased A β production. It is also possible the activation of JNK could induce apoptosis. Alternatively, MAPK4 could alter the phosphorylation state of APP which has been shown to affect its preferential processing by the β - and α -secretase by affecting the trafficking of APP and disrupting PS1/ β -catenin interactions (Hung and Selkoe 1994), (Walter, Capell et al. 1997). Additionally, the phosphorylation state of the cells has also been shown to be critical for PS1 activity (Seeger, Nordstedt et al. 1997). JNK activation and protein phosphorylation are also several other pathways MAPK4 could be acting to increase A β production.

Another gene identified in the screen is Calmodulin. Calmodulin is a loop-helix-loop Ca²⁺-binding protein that transduces Ca²⁺ signals by interacting with specific target proteins

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including CaMKII, CaMKIV, calcineurin, spectrin A2, p21, and neuronal nitric oxide synthase (Means 1981). When Ca^{2+} binds Calmodulin it undergoes a conformational change that allows it to bind to target proteins and to stimulate or inhibit their activities. Calmodulin has been shown to regulate A β production in cell-free preparations and intact cells using the Calmodulin antagonists W-7 and trifluoperazine. These known inhibitors of Calmodulin activity can also inhibit A β production (Desdouits, Buxbaum et al. 1996). Thus, intracellular Ca^{2+} concentrations and/or calmodulin target proteins can affect APP processing. This compound data validates the fact that an increase in A β production is observed when Calmodulin is overexpressed.

Ca^{2+} dysregulation, particularly a drop in cytoplasmic Ca^{2+} levels, is concomitant with an increase in A β production associated with PS1 FAD mutations (Yoo, Cheng et al. 2000). PS1 FAD mutations can significantly attenuate capacitative calcium entry (CCE) and store depletion-activated currents suggesting reduced CCE can increase A β generation (Yoo, Cheng et al. 2000). The overexpression of the calmodulin gene found in the screen could also cause a drop in intracellular calcium levels, and could prevent adequate CCE. It is likely the decrease in intracellular Ca^{2+} could increase PS1 activity, either directly or indirectly, resulting in more A β secretion from cells. Data described in detail below suggests that SfA potently inhibits A β 40 & A β 42 production and inhibits C99 and Notch cleavage suggesting gamma secretase activity is linked to Ca^{2+} homeostasis through the immunophilin protein CyD.

Another gene, human acid ceramidase catalyzes the hydrolysis of ceramide to sphingosine and fatty acid (Ferlinz, Kopal et al. 2001). Ceramide serves as the precursor for most sphingolipids and is a signaling molecule that induces apoptosis in a number of different cell types, typically through caspase-3 activation. Accumulating ceramide levels are associated with Alzheimer's disease and are thought to be part of the oxidative neurotoxic pathway in the aging AD brain. Overexpression of ceramidase increases A β production linking ceramidase cleavage to A β . Since ceramide seems to act through apoptosis it is likely the acid ceramidase is increasing apoptosis as well.

The ceramide status of the cell has been shown to regulate both BACE stability and A β biogenesis (Puglielli, Ellis et al. 2003). Previous studies demonstrate that high levels of ceramide can increase A β secretion in an apoptosis independent fashion (Puglielli, Ellis et al. 2003). Ceramidase overexpression would decrease ceramide levels and increase sphingosine and free fatty acid levels (FFA) possibly altering membrane fluidity, a process

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known to alter tau phosphorylation and A β polymerization (Wilson and Binder 1997). Although the mechanism is unclear, it could be that ceramidase can affect the FFA levels leading to altered APP processing and more A β production and/or secretion.

TOB3 is a AAA-ATPase that performs chaperone-like functions that assist in the assembly, operation, or disassembly of protein complexes, including protein secretion (Strausberg, Feingold et al. 2002). This class of proteins helps to maintain the integrity of the endoplasmic reticulum (ER) as proteins are being constitutively processed. In the absence of AAA-ATPase activity there is excessive accumulation of misfolded proteins causing ER expansion and cell death (Kobayashi, Tanaka et al. 2002). The most likely explanation of TOB3 overexpression increasing A β secretion is linked to its ability to effect protein folding and trafficking in the ER. If this process is stimulated, as with TOB3 overexpression, it is possible APP processing would be increased.

Our screening data has shown that TOB3 overexpression alters APP processing leading to more A β production and fewer C99 & C83 C-terminal fragments. TOB3 expression also decreases APP and sAPP α levels in N2A cells to a greater extent than HEK 293 cells (data not shown). This suggests that TOB3 affects one or more components of the APP processing machinery resulting in increased APP and C99 cleavage. Since TOB3 is involved in the transport and processing of proteins, the exact mechanism and specificity of TOB3 on APP processing is currently being determined.

Carboxypeptidase Z (CPZ), another cDNA identified from the functional screen, is a member of the metallo-carboxypeptidase gene family along with CPE and CPD which are thought to function in the intracellular processing of bioactive peptides and proteins prior to their secretion. CPZ is a unique carboxypeptidase because it contains a functional N-terminal cysteine-rich frizzled domain that binds to Wnt and wingless proteins which are a critical component of Wnt signal transduction (Moeller, Swindell et al. 2003).

Another interesting cDNA is cyclophilin D (CyD, also known as CyF. Reference to nomenclature can be found in Current Medicinal Chemistry, 2003, 10, 1485-1506 1485 Cyclophilin D as a Drug Target, Waldmeier, et al). CyD was found to be involved in A β production. Cyclophilin proteins, such as CyD, are peptidyl prolyl isomerases involved in protein trafficking and maturation. Cyclophilins are primarily localized to the cytoplasm except CyD which is exclusively localized in the mitochondrial matrix (Waldmeier, Zimmermann et al. 2003). CyD when overexpressed, could stabilize endogenous PS1 N-terminal fragments (NTFs). A common lesion of the AD brain is the presence of intracellular

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neurofibrillary tangles made up of abnormally phosphorylated tau, a microtubule-associated protein (Johnson and Bailey 2002). When overexpressed with APP, CyD could increase caspase-3 activity, indicating a possible mechanism through which A β secretion is modulated. These results suggest that when overexpressed, CyD is an important factor required for the cleavage of APP and C99 through the γ -secretase pathway.

In one aspect, CyD is an integral constituent of the mitochondrial permeability transition pore thought to bind the adenine nucleotide translocator and regulate pore opening (Waldmeier, Zimmermann et al. 2003). Permeabilization of mitochondrial membranes is a consequence of cellular stress that leads to dissipation of the mitochondrial membrane potential, release of apoptogenic proteins, and culminates in apoptotic cell death (Waldmeier 2002).

Mitochondrial failure has been suggested to play a significant role in the development of AD neuropathology in Down's syndrome patients, by promoting aberrant β -APP processing and intracellular accumulation of A β (Busciglio 2002). Increases in intracellular calcium levels has also been shown to elicit accumulation of intracellular A β (LaFerla 2002). Surprisingly, full length APP does not only traffic along the secretory pathway, but it is also targeted to mitochondria of cultured cortical neuronal cells in the brain of a transgenic mouse model for AD. Incomplete translocation and progressive accumulation of β -APP on mitochondrial membranes can lead to mitochondrial dysfunction and can also play a role in the pathogenesis of AD (Anandatheerthavarada, Biswas et al. 2003). It is known that overexpression of mitochondrial proteins can lead to the formation of insoluble aggregates within the matrix (e.g. uncoupling protein-3, UCP3). These aggregates can both derange the normal function of mitochondria and increase the passive permeability of the inner membrane. CyD may play an important role in the mitochondria dealing with excess APP or C-terminal fragment processing.

CyD is known target of the immunosuppressant drugs such as cyclosporin. These compounds block mitochondrial permeability transition (MPT) and prevent apoptosis (Samantha J. Clarke 2002; Waldmeier 2002). FK506 compounds are also known to bind immunophilin proteins but do not bind CyD (Uchino 2003).

A cyclosporin is considered as binding to cyclophilin if it binds to human recombinant cyclophilin at least one fifth as well as does Cyclosporin (also referred to as cyclosporin A) in

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the competitive ELISA test described by Quesniaux in Eur. J. Immunol. 1987, 17, 1359-1365. In this test, the cyclosporin to be tested is added during the incubation of cyclophilin with coated BSA-Ciclosporin and the concentration required to give a 50% inhibition of the control reaction without competitor is calculated (IC_{50}). The results are expressed as the Binding Ratio (BR), which is the log to the base 10 of the ratio of the IC_{50} of the test compound and the IC_{50} in a like test using Ciclosporin in place of the test cyclosporin. Thus a BR of 1.0 indicates that the test compound binds cyclophilin one factor of ten less well than does Ciclosporin, and a negative value indicates binding stronger than that of Ciclosporin.

The cyclosporins active as neuroprotective agents have a BR lower than 0.7, (since $\log_{10} 5 = 0.7$ approx), preferably equal to or lower than zero.

A cyclosporin is considered to be non-immunosuppressive when it has an activity in the Mixed Lymphocyte Reaction (MLR) of no more than 5%, preferably no more than 2%, that of Ciclosporin. The Mixed Lymphocyte Reaction is described by T. Meo in "Immunological Methods", L. Lefkovits and B. Peris, Eds., Academic Press, N.Y. pp. 227-239 (1979). Spleen cells (0.5×10^6) from Balb/c mice (female, 8-10 weeks) are co-incubated for 5 days with 0.5×10^6 irradiated (2000 rads) or mitomycin C treated spleen cells from CBA mice (female, 8-10 weeks). The irradiated allogeneic cells induce a proliferative response in the Balb c spleen cells which can be measured by labelled precursor incorporation into the DNA. Since the stimulator cells are irradiated (or mitomycin C treated) they do not respond to the Balb/c cells with proliferation but do retain their antigenicity. The IC_{50} found for the test compound in the MLR is compared with that found for Ciclosporin in a parallel experiment.

It has been found that compounds which are judged as non-immunosuppressive in the MLR above are often inactive in an IL-2 Reporter Gene Assay, and thus an IL-2 Reporter Gene Assay may be used, e.g. as a primary screen, for selection of non-immunosuppressive, cyclophilin-binding cyclosporin compounds for use in the invention.

The non-immunosuppressive, cyclophilin-binding cyclosporin compounds which are active as agents to treat pathological conditions associated with A β secretion, e.g. as inhibitors of extracellular accumulation of amyloid plaques in AD are hereinafter referred to as Active Compounds.

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The active compounds are therefore useful in the treatment of any clinical condition involving A β peptide secretion, expression of endogenous PS1 N-terminal fragments (NTFs) or increased gamma-secretase activity. Further the active compounds are useful in the treatment of conditions involved in apoptosis that increase A β secretion. A β peptide formation and subsequent aggregation are not only a hallmark of AD, but are also an integral part of other neurological disease such as Parkinson's, Huntington's, and other systemic amyloidosis (Selkoe 1989; Price, Borchelt et al. 1993; Citron, Vigo-Pelfrey et al. 1994). It is clear from these results that apoptosis and A β production are integrally linked.

The active compounds also have utility to modulate "peripheral A β modifiers" not expressed in the brain. Peripheral amyloidosis can result in such phenotypes as cardiac and dermatological amyloidosis (Yamaguchi, Yamazaki et al. 1992), (Selkoe 1989). If a key regulator of peripheral A β is found, it is also possible to derive novel therapeutics such to such targets which prevent the need to penetrate the blood brain barrier. A drop in peripheral A β levels has been shown to decrease levels of A β in the transgenic mouse brain, resulting in less plaque formation (Bohrmann, Tjernberg et al. 1999; DeMattos, Bales et al. 2002).

It is found that many of the Active Compounds have structures differing from that of Ciclosporin specifically at the 4 and/or 5 positions. Other positions at which the structures of the Active Compounds may differ from that of Ciclosporin are positions 6 and 7.

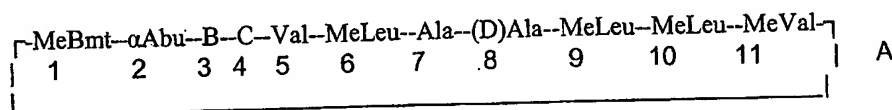
One group of Active Compounds are cyclosporins in which the MeLeu group at position 4 is replaced by a different N-methylated amino acid for example γ -hydroxy-MeLeu, Melle, MeVal, MeThr, MeAla, Me Tyr or MeTyr(O-PO(OH)₂), or Pro. In addition to Melle and MeThr, the allo-forms Mealle and MeaThr may also be used. In the allo-form, the stereochemistry at the β -position has the opposite configuration to that of the natural amino acid, so that the normal form and the allo-form constitute a pair of diastereoisomers.

A further group of Active Compounds is that in which Val at the 5-position is replaced by an N-alkyl-, preferably N-methyl-, amino acid. Preferably the amino acid which is N-alkylated is Val or Leu. Preferably the hydrogen of the imino group of [Val]⁵ is replaced by a non-branched C₁₋₆alkyl group, preferably methyl, ethyl or n-propyl, particularly methyl. The latter preferred group of Active Compounds are all novel.

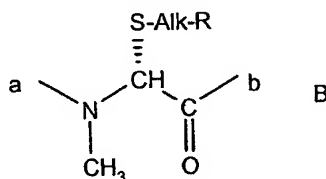
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Additionally or alternatively, certain Active Compounds may differ from Ciclosporin at the 1, 2, 3, and/or 6 positions.

A particular class of Active Compounds for use in the present invention are Ciclosporin derivatives of formula A



wherein B is an amino acid residue of formula B



wherein a denotes the bond to the αAbu residue in position 2;

b denotes the bond to the residue C in the 4 position;

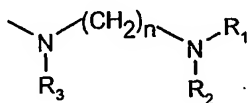
Alk represents straight or branched chain alkylene containing from 2 to 6 carbon atoms or cycloalkylene containing from 3 to 6 carbon atoms, and

R represents

a carboxy or alkyloxycarbonyl radical;

a radical $-\text{NR}_1\text{R}_2$ in which R_1 and R_2 are the same or different and represent hydrogen, alkyl, C_{2-4} alkenyl, C_{3-6} cycloalkyl, phenyl (optionally substituted by halogen, alkoxy, alkyloxycarbonyl, amino, alkylamino or dialkylamino) or a benzyl or saturated or unsaturated heterocyclyl radical containing 5 or 6 ring atoms and 1 to 3 heteroatoms, or in which R_1 and R_2 form together with the nitrogen atom to which they are attached a saturated or unsaturated heterocycle containing 4 to 6 ring atoms and optionally containing a further heteroatom selected from nitrogen, oxygen or sulphur and optionally substituted by alkyl, phenyl or benzyl;

a radical of formula



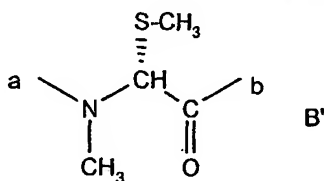
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wherein R_1 and R_2 are as defined above, R_3 represents hydrogen or an alkyl radical and n is a whole number from 2 to 4,

and wherein alkyl denotes straight or branched chain alkyl containing from 1 to 4 carbon atoms;

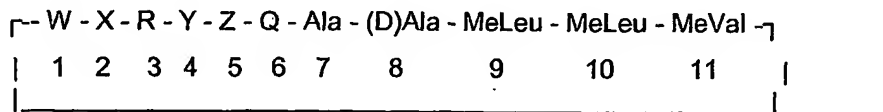
C is MeLeu or 4-hydroxy-MeLeu; and the pharmaceutically acceptable salts thereof.

This class of Ciclosporin derivatives is further described in published International patent applications Nos. WO 98/28328, WO 98/28329 and WO 98/28330. A particularly preferred compound of this class is the compound of formula A in which B is the amino acid residue B'



and C is the amino acid residue 4-hydroxy-MeLeu.

A particularly preferred group of Active Compounds is constituted by the compounds of Formula I:



in which W is MeBmt, dihydro-MeBmt or 8'-hydroxy-MeBmt;

X is α Abu, Val, Thr, Nva or O-methyl threonine (MeOThr);

R is Sar or (D)-MeAla;

Y is MeLeu, γ -hydroxy-MeLeu, Melle, MeVal, MeThr, MeAla, Me Tyr, MeTyr(O-PO(OH)₂),

Mealle or MeaThr, or Pro;

Z is Val, Leu, N-Alk-Val or N-Alk-Leu

wherein Alk represents Me or Me substituted by

vinyl optionally substituted by

phenyl, or an N S or O heteroaryl containing 6 ring members, or

phenyl optionally substituted by

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halogen; and

Q is MeLeu, γ -hydroxy-MeLeu or MeAla and the pharmaceutically acceptable salts thereof.

The groups W,X,Y,Z and Q have, independently, the following preferred significances:

W is preferably W' where W' is MeBmt or dihydro-MeBmt;

X is preferably X' where X' is α Abu or Nva, more preferably X'' where X'' is α Abu;

Y is preferably Y' where Y' is γ -hydroxy-MeLeu, MeVal, MeThr, MeAla or MeTyr(O-PO(OH)₂);

Z is preferably Z' where Z' is Val or MeVal; and

Q is preferably Q' where Q' is MeLeu;

One especially preferred group of Active Compounds are the compounds of Formula I in which W is W', X is X', Y is Y', Z is Z' and Q is Q'.

Particularly preferred Active Compounds of Formula I are:

- a) [dihydro-MeBmt]¹-[γ -hydroxy-MeLeu]⁴-Ciclosporin,
- b) [MeVal]⁴-Ciclosporin,
- c) [Melle]⁴-Ciclosporin,
- d) [MeThr]⁴-Ciclosporin,
- e) [γ -hydroxy-MeLeu]⁴-Ciclosporin,
- f) [Nva]²-[γ -hydroxy-MeLeu]⁴-Ciclosporin,
- g) [γ -hydroxy-MeLeu]⁴-[γ -hydroxy-MeLeu]⁶-Ciclosporin,
- h) [MeVal]⁵-Ciclosporin,
- i) [MeOThr]²-[(D)MeAla]³-[MeVal]⁵-Ciclosporin,
- j) [8'-hydroxy-MeBmt]¹-Ciclosporin,
- k) [MeAla]⁶-Ciclosporin,
- l) [DMeAla]³-[MeTyr(OPO(OH)₂)]⁴-Ciclosporin,
- m) [N-Benzyl-Val]⁵-Ciclosporin,
- n) [N-5-Fluoro-Benzyl-Val]⁵-Ciclosporin,
- o) [N-Allyl-Val]⁵-Ciclosporin,
- p) [N-3-Phenyl-Allyl-Val]⁵-Ciclosporin,
- q) [Pro]⁴-Ciclosporin

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Especially preferred Active Compounds are [Melle]⁴-Ciclosporin and [γ -hydroxy-MeLeu]⁴-Ciclosporin, most especially [Melle]⁴-Ciclosporin.

In addition to the compounds of Formula I, preferred Active Compounds include, for example

r) [γ -hydroxy-MeLeu]⁹-Ciclosporin.

The Active Compounds may be obtained by methods including:

- 1) Fermentation
- 2) Biotransformation
- 3) Derivatisation
- 4) Partial Synthesis
- 5) Total Synthesis.

These methods are described generally and more specifically in Examples 1 to 10 of EP 0484281 B and US Pat. No. 5767069. This general description and the teaching of these Examples are incorporated by reference in the present application. Example 11 of EP 0484281 B describes measurement of the immunosuppressive and cyclophilin-binding activities of representative Active Compounds relative to Ciclosporin, and the teaching of this example is also included within the disclosure of the present application.

Thus the invention provides use of a nonimmunosuppressive, cyclophilin-binding cyclosporin in the manufacture of a medicament for treating or preventing pathological conditions associated with AB secretion such as Alzheimer's Disease, Parkinson's Disease, tauopathies, prion diseases, frontotemporal dementia, striatonigral degeneration, Lewy body dementia, Huntington's disease, Pick's disease, amyloidosis, and other neurodegenerative disorders associated with excess AB production.

The invention further provides a method for the treatment or the prevention of preventing pathological conditions associated with AB secretion such as Alzheimer's Disease, Parkinson's Disease, tauopathies, prion diseases, frontotemporal dementia, striatonigral degeneration, Lewy body dementia, Huntington's disease, Pick's disease, amyloidosis, and other neurodegenerative disorders, comprising administering to said patient an effective amount of an Active Compound of the invention.

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The Active Compound may be administered by any conventional route, in particular enterally, e.g. orally, for example in the form of solutions for drinking, tablets or capsules or parenterally, for example in the form of injectible solutions or suspensions. By the intravenous route an indicated daily dosage may be from 1 to 20 mg/kg, preferably from 3 to 10 mg/kg, and by the oral route from 1 to 50 mg/kg, preferably from 10 to 30 mg/kg.

The toxicity of the Active Compounds is believed to be less to that of Ciclosporin. As the Active Compounds are not immunosuppressive, certain side effects of Ciclosporin related to immunosuppression are avoided. Other side effects associated with Ciclosporin, particularly nephrotoxicity and central nervous system toxicity in long term use, are conveniently less than with Ciclosporin.

Preferred galenic formulations for the Active Compounds include those based on microemulsions as described in British Patent Application 2 222 770A, which include topical as well as oral forms; also oral and injectable forms obtained from solid solutions comprising a fatty acid saccharide monoester, e.g. saccharose monolaurate, as described in British Patent Application 2 209 671A. Suitable unit dosage forms for oral administration comprise e.g. from 25 to 200mg Active Compound per dosage.

Formulation Examples A, B, C and D of EP 0484281 B are incorporated herein by reference:

The individual components of these formulations, as well as the methods for their preparation, are fully described in British Patent Application 2 222 770, the contents of which are incorporated herein by reference.

The usefulness of the active compounds as neuroprotective agents may be demonstrated in vivo or in vitro tests, e.g:

The active compounds of the invention can be provided alone, or in combination, or in sequential combination with other agents. For example, the active compounds of the invention can be administered in combination with anti-inflammatory agents such as but not limited to corticosteroids following stroke or spinal cord injury as a means for blocking further neuronal damage and inhibition of axonal regeneration, Neurotrophic factors such as NGF, BDNF or other drugs for neurodegenerative diseases such as Exelon™ or Levodopa. As

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used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

The structure of the active ingredients identified by code nos., generic or trade names may be taken from the actual edition of the standard compendium "The Merck Index" or from databases, e.g. Patents International (e.g. IMS World Publications). The corresponding content thereof is hereby incorporated by reference. Any person skilled in the art is fully enabled to identify the active ingredients and, based on these references, likewise enabled to manufacture and test the pharmaceutical indications and properties in standard test models, both *in vitro* and *in vivo*.

For the indications mentioned above, the appropriate dosage will, of course, vary depending upon, for example, the particular molecule of the invention to be employed, the mode of administration and the nature and severity of the condition being treated.

EXAMPLES

The following methods are performed to conduct the examples disclosed below:

Transfections. CHO K1 cells (ATCC, Manassas, VA) are plated with DMEM, 10% Fetal Bovine Serum, 5% Penn/Strep, and 22mg of L-Proline (Sigma Chemical, St. Louis, MO). The plates are incubated overnight at 37°C in water jacked CO₂ cell culture chambers. The cDNA of interest is co-transfected with full length APP in a 1:15 ratio (cDNA:APPwt(695)) using Qiagen® SuperFect reagent is used according to the manufacturer's directions. In 6-well dishes, 5x 10⁵ cells are plated with DMEM, 10% Fetal Bovine Serum, 5% Penn/Strep (Sigma Chemical, St. Louis, MO) and grown up for 24 hours. The SuperFect mix is made up with 100µl of serum free medium (DMEM), 3µg of total DNA, and 20µl of SuperFect. The media is removed from the cells and 1mL of fresh media is added. The entire SuperFect mix is added to the media and incubated at 37°C for 2 hours. The mixture is then removed and the cells are washed once with 3mL of PBS. Fresh media is added back to the cells and they are incubated for 24 or 48 hours.

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Immunocytochemistry – CHO cells are transfected in OPTIMEM (serum-free media) with appropriate constructs using Lipofectamine as described by manufactures (Life Technologies). The CHO cells are immunostained and immunoreactivity is observed as described in Dev et al 1999, *Neuropharmacol.*, (1999) 38, 635-644. Flag-protein expression is detected using anti-Flag M2 monoclonal mouse antibody (Sigma), the secondary antibody is Texas Red-X goat anti-rabbit IgG (Molecular Probes).

Flow Cytometry analysis of caspase-3. HEK cells are transiently transfected with CyD or CPZ along with APPwt for 24 hours. Cells are fixed, permeabilized, and stained using the Caspase-3 apoptosis kit from BD Biosciences (#550914). FITC stained cells are gated from $\geq 10^1$ and measured as cells positive for active caspase-3. Forward and side light scatter are also examined to check the health of the cell population.

Compound treatment. HEK 293 cells stably expressing the APPswe mutant are treated for 24 hours with cyclosporin A, Sangliferhin A, FK506 (as described in Sedrani, et al., J. Am. Chem. Soc. 125, pp 3849-3859 (2003) incorporated by reference in its entirety) and N-methyl-4-valine-cyclosporin concentrations. Cell viability is measured using the CellTiter-Glo Kit from Promega (#G7573). A β 40 & 42 levels are measured in the cell supernatants using an in house two-sandwich ELISA (the in-house ELISA is the same format as described except antibodies developed at Novartis are used instead of the Biosource antibodies). A cell line stably expressing a 5xGal4 response element-luciferase reporter and a C99-Gal4-VP16 or a Notch-Gal4-VP16 construct is used to measure C99 or Notch cleavage (as disclosed in Maltese, Wilson et al. 2001). When cleaved by γ -secretase, Gal4-VP16 is released and binds to the reporter increasing luciferase activity. The stable cells are treated with the compounds and the IC₅₀ values are determined.

A β ELISA. A commercially available mouse monoclonal antibody directed to the NH₂ terminus of the A β peptide is used as the capture antibody in pre-coated 96 well plates (Biosource Cat#KBH3481/PPO81 for A β 40 and Cat#KBH3441/PPO81 for A β 42). Polyclonal detection antibodies are obtained from Biosource (anti-hA β 40 Cat#44-348 and anti-hA β 42 Cat#44-344) and diluted 1/220 in 15mM sodium azide. The secondary antibody (Biosource Cat#KBH3481 for A β 40 and Cat#KBH3441 for A β 42) is a horseradish peroxidase labeled anti-rabbit IgG. The secondary antibody is diluted 1/100 in 3.3mM thymol. The antibody-

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coated plates are washed 4x in PBS-TE (1mM EDTA and 0.05% Tween 20, wash buffer) on a microplate washer (Biotek Instruments, Inc, Winooski, VT) prior to use. 100 µl of the transfected cell's conditioned media is removed and diluted 1:2 in sample diluent containing 1mM AEBSF (Biosource, Camarillo, CA). 100 µl of this mixture is added to the washed, antibody coated 96 well plate, covered with tape, and incubated at 4°C overnight. The samples are removed and the plates are washed 4x with wash buffer. Detection antibody solution is added at 100µl/well and the plates are incubated at room temperature for 2 hours while shaking. The plates are washed again 4x with wash buffer and the secondary antibody solution is added at 100 µl/well and incubated for 2 hours while shaking. The plates are washed 5x in wash buffer and patted dry on a paper towel. 100 µl of stabilized chromogen (tetramethylbenzidine) is added to each well and the plate is incubated for 30 minutes in the dark. 100 µl of stop solution (1N H₂S) is added to the plates to stop the reaction. The plates are read on a microplate reader at 450 nM (Molecular Devices) within one hour.

Antibodies and Western blot analysis. The cDNA for APP wild type and the APP Swedish mutant are inserted into the pCI plasmid expression vector downstream of a cytomegalovirus promoter as previously described (Promega, Madison, WI) (Bodendorf, U., Fischer, F., Bodian, D., Multhaup, G., Paganetti, P. 2001 *J. Biol. Chem.* 276:12019-12023).

PS1 NTF antibody recognizes the N-terminus of PS1 (Thinakaran, Borchelt et al. 1996).

Cultured HEK 293 cells are extracted at 24hr post-transfection in RIPA buffer (10 mM Tris, pH 7.5, 150 mM sodium chloride, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium-deoxycholate, 1% SDS) containing protease inhibitors (Complete® Roche Molecular Biochemicals) and centrifuged at 4 °C for 10 min at 10,000 x g. The supernatants are collected and the pellets discarded. Subsequently, the cell extracts were resolved by SDS polyacrylamide gel electrophoresis, transferred to PVDF Immobilon-P® membranes (Millipore), and probed with primary antibodies as indicated. Immunological detection is carried out with the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ) as previously described (Manni, M., et al., 1998. *FEBS.* 427:367-370).

Immunocytochemistry – CHO cells are transfected in OPTIMEM (serum-free media) with appropriate constructs using Lipofectamine as described by the manufacturer (Life Technologies). The CHO cells are immunostained and immunoreactivity is observed as described previously (Dev et al 1999, *Neuropharmacol.*, (1999) 38, 635-644). Flag-protein

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expression is detected using anti-Flag M2 monoclonal mouse antibody (Sigma), the secondary antibody is Texas Red-X goat anti-rabbit IgG (Molecular Probes).

CPZ Constructs- Wild-type human Carboxypeptidase (hCPZ) is obtained from a normalized cDNA library of pure human DRG (L0001) purchased from Life Technologies, Inc (LTI, Catalog Number: 11315-017, Lot Number: 81027-242). The cDNA inserts are cloned into the EcoR V and Not I sites in 5' to 3' direction into the Gateway-compatible vector, pCMV-SPORT6. Deletion of the frizzled (FZ) domain corresponding to amino acids 42-161, the catalytic (Cat) domain from amino acid 179-568, the point mutant (amino acid E251A), and introducing of a N-terminal FLAG-tag (DYKDDDDK Seq ID. No 1) after amino acid 29 is done by site-directed mutagenesis using primers:

5' CCTCCAGGCCTCCCCGAAGCTTCTCGGCGCTGTCTGCAGCTGGTGGCCTGTGG-3'
(Seq Id No. 2) and

5' CCACAGGCCACACAGCTGCAGACAGCGCCGAGAAGCTTCGGGGAGGCCTGGAGG-3'
(Seq Id No. 3) for deletion of the frizzled domain,

5' CCACACGGCCAGCCCTCTTCATCCGGGCCAGCCCTGAGGGCAGTGCCTCGTCAGC -
3' (Seq Id No. 4) and

5' GCTGACGAGGCACTGCCCTCAGGGCTGGCCCGGATGAAGAGGGCTGGCCGTGTGG
3' (Seq Id No. 5) for deletion of the catalytic domain,

5' GCATCTCCCGGCCCCGCCACCGCGTTGCCATGAATGTTGC-3' (Seq Id No. 6) and

5' GCAACATTCATGGCAACGCGGTGGCGGGCCGGGAGATGC-3' (Seq Id No. 7) for the point mutation E251A, and

5'GGTGGCCTGTGGCATTACCCCTTGTCATCGTCGTCCTTGTAGTCGGCGGGGTTCGCG
TCAAACCTCG-3' (Seq Id No. 8) and

5'CGAGTTTGAGCGGAACCCCGCCGACTACAAGGACGACGATGACAAGGGTGAATGCCA
CAGGCCACC-3' (Seq Id No. 9) for introducing the FLAG-tag. Isolation of all DNA from transformed *E.coli* is performed using Qiagen plasmid kits. The sequence of all open reading frames is verified by DNA sequencing using the ABI Prism 3700 DNA Analyzer system.

In situ hybridizations- Using polymerase chain reaction (PCR) with self-priming oligonucleotide primers flanked in 5' with SP6- and T7-promotor recognition sequences, it is possible to generate riboprobe templates from any known gene sequence without any cloning step. PCR reaction is carried out for 40 cycles with denaturation step at 95°C for 45 sec,

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annealing phase at 58°C for 30 sec and extension phase at 70°C for 1 min. After separation on 4 % agarose gel, the PCR products are cleaned with QiAQuick purification kit according to the manufacturer's instructions (Qiagen, Switzerland). The cleaned PCR product is transcribed using T7-RNA polymerase (anti-sense) and SP6-RNA polymerase (sense) at 37°C for 2 hours using dNTP containing Digoxigenin-UTP. Un-incorporated nucleotides are removed, the probe is ethanol-precipitated and dissolved in 50 µl water before storage at -20°C. Serial dilutions of the DIG-probe and labeled control RNA (known concentration) are spotted onto a nylon membrane. Incubation with alkaline phosphatase conjugated anti-DIG antibody is followed by color development phase using NBT/BCIP (75 mg/ml Nitro Blue Tetrazolium in 70% dimethyl formamide and 30% water and 50 mg/ml 5-Bromo-4-Chloro-3-Indoyl Phosphate in 100% dimethyl formamide), as substrate for the alkaline phosphatase. The CPZ probe concentration is estimated by comparison of the spot intensities with the labeled control RNA. ISH is performed using the fully automated instrument Discovery™ (Ventana Medical Systems, Strasbourg) for in situ hybridization and immunochemistry. The protocol performed to localize this gene using paraffin-embedding tissue section is established within the RNA analytics laboratory and described as below, Deparaffinization and rehydration of tissues sections is performed on solvent-free conditions using EZprep solution (Ventana Medical Systems SA, Strasbourg) for 8 min at 75°C followed by 8 more min at 42°C. All pretreatment steps are done with the RiboMap™ kit (Ventana Medical Systems SA, Strasbourg) following the manufacturer's instructions with one additional permeabilization step using enzymatic digestion : optimal results are obtained with 12 µg/ml of proteinase K at 37°C for 16 min. CPZ probe hybridization is performed at 45°C for 6 hours with adapted quantities of DIG-riboprobe (CPZ probe = 5 ng/slide) diluted in RiboHybe solution (Ventana Medical Systems SA, Strasbourg). Post-hybridization washes are performed at 50°C for 8 min on high stringency conditions (0.1 x SSC) for 3 times. For the detection of DIG-label, a biotin-conjugated mouse anti-digoxin antibody (Jackson ImmunoResearch Inc.) is applied for 30 min at 37°C after dilution at 1/2000 in antibody diluent and followed BCIP/NBT chromogenic detection is done using BlueMap™ Kit (Ventana Medical Systems SA, Strasbourg) according to manufacturer's instructions. The substrate incubation time for optimal signal / noise balance is 4 hours. Counter staining using ISH nuclear fast red is performed for 10 min. Sections are mounted in Crystal mount and post-mounted using Permount.

Example 1

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Presenilin Processing: The observation that the overexpression of cDNAs increased A β levels with the C99 substrate suggests an increase in GACE mediated cleavage of APP. Since CPZ and CyD significantly increased the processing of C99 and increased A β 42 secretion, they are chosen for further investigation. PS1 N-terminal fragment (NTF) levels were examined in HEK 293 cells transfected with CPZ or CyD. In cells stably transfected with PS1, both full length PS1 and NTFs are easily detectable. In contrast, in non-transfected HEK cells, the endogenous full length PS1 and the NTF levels are low. In cells overexpressing CPZ or CyD, there is a clear increase in PS1 NTF levels indicating these fragments are being generated at higher rates or the endogenous NTFs are being stabilized over the 48 hr transfection period.

Analysis of CPZ: CPZ is a member of the Carboxypeptidase (CP) gene family, in the CPE subfamily, known to process bioactive neuropeptides (Song and Fricker 1997). CPE related enzymes are generally involved in selective processing reactions, by the selective removal of basic residues from the C-terminus of processing intermediates. To determine whether the catalytic activity, expression, or the subcellular localization of CPZ was required to induce A β secretion, three mutant constructs of CPZ are generated. In the first construct, the catalytic domain is completely removed (Δ cat) leaving only the signal peptide, frizzled domain, and C-terminus. In the second construct, the frizzled domain (Δ fz) is deleted, and in the third construct, a single point mutation (Glu251Ala) is introduced into the catalytic domain that would impair the catalytic activity of CPZ. Each CPZ variant is co-transfected into HEK 293 cells with either the APPwt or C99 substrates. Each of the three mutant constructs have no activity as compared to wild type CPZ or the flag tagged CPZ. The results are similar with both the APPwt and C99 substrates.

A possible explanation for the lack of activity of the CPZ mutants is the lack of expression or mislocalization of the protein in the cell. To address this question, the CPZ mutants are flag tagged and overexpressed in CHO cells. In permeabilized cells, CPZ wild-type has a clear plasma membrane or late secretory vesicle localization. This result is consistent with previous studies examining the intracellular distribution of CPZ (Novikova, Reznik et al. 2000). The subcellular distribution remained unchanged for the CPZ mutant constructs; Δ cat, Δ fz, and Glu251Ala. These findings suggested that the catalytic activity of CPZ, not its intracellular localization or level of expression is required for the induction of A β levels in CHO cells.

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Disruption of CPZ catalytic activity abolished A β production without affecting expression or subcellular distribution. The overexpression of CPZ could compete with Wnt binding to endogenous frizzled receptors resulting in less β -catenin activation (Tesco, Kim et al. 1998). A decrease in β -catenin activity has been associated with increased A β production, thought to be mediated by the ability of PS1 to increase β -catenin association with GSK3 β (Kang, Soriano et al. 1999). It is possible that CPZ can induce A β production by activating PS1 cleavage via caspase-3, resulting in less PS1/ β -catenin complexes (Tesco, Kim et al. 1998). If PS1 is not bound by β -catenin then it can more readily associate with the GACE complex. Since frizzled domains are characterized as protein interaction domains, it is possible that the frizzled domain in CPZ could interact with a component of the γ -secretase complex, i.e.-PS1, or APP itself. These interactions are currently being investigated.

Interestingly, the C-terminus of CPZ contains a putative furin cleavage site thought to release CPZ from the plasma membrane into the extracellular milieu (Novikova, Reznik et al. 2000). Furin is also involved in the release of the Notch ligand Delta from Notch at the cell surface (Ikeuchi and Sisodia 2003). Although the furin site in CPZ has not been confirmed as a furin substrate, it has been demonstrated that CPZ can be secreted from cells (Novikova, Reznik et al. 2000). This suggested that CPZ could be in the same processing pathways as Notch, but how furin cleavage of CPZ is related to A β production is currently unclear (Kim, Wang et al. 1999). However, the observation that CPZ, like CyD overexpression, can drive cells into apoptosis, indicates a common mechanism for the two proteins affecting A β production.

Previous findings (Novikova and Fricker 1999) suggest that CPZ is expressed in rat brain leptomeningeal cells but CPZ has not been shown to be expressed in regions of the brain known to be affected by Alzheimer's disease (Novikova and Fricker 1999). To determine where CPZ is expressed, mouse brain slices are analyzed by *in situ* hybridization. In wild type C7BL-6 mice (Jackson Laboratories), a CPZ anti-sense probe exhibited binding as a broad distribution in the brain with the cerebellum and frontal cortex having the highest level of signal. Upon closer examination, CPZ is also found to be expressed in the hippocampus near the CA1 & CA3 regions, most likely in the pyramidal cells. In the cerebellum, CPZ is expressed proximal to the molecular region in the Perkinje and granular cell layers. The distribution of CPZ in the frontal cortex did not seem to be localized to a specific cell type, but instead, evenly expressed across the region. The localization of CPZ in

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the mouse hippocampus, cerebellum, and frontal cortex demonstrates its expression in regions of the brain known to be affected by Alzheimer's disease in humans.

Characterization of CyD: To test whether these immunophilin-binding compounds could modulate APP processing, HEK/APPSwe stable cells are treated with a range of concentrations of cyclosporin A, Sangliferhin A, FK506 or N-methyl-4-valine-cyclosporin concentrations. For each concentration, viability is measured by an MTS assay according to manufacturers instructions (Promega Cat# G5421) and secreted A β 40 & A β 42 is measured using the ELISA assay described herein. The viability IC₅₀ for all of the compounds is >40 μ M. The inhibitory IC₅₀ for cyclosporin A, Sangliferhin A is < 3 μ M for A β 40 & A β 42 indicating a strong inhibition of A β secretion. The IC₅₀ for N-methyl-4-valine-cyclosporin is < 10 μ M for A β 40 & A β 42 secretion, while FK506 had no effect on A β 40 secretion. In contrast, FK506 treatment causes a dose dependent increase in A β 42 from 3-20 μ M.

It has been demonstrated that immunosuppressants such as cyclosporin A, can inhibit neurodegeneration and apoptosis in several animal models and inhibit A β induced mitochondrial damage in isolated mouse mitochondria (Kim 2002). Since CyD is a target of cyclosporin A and localized to the mitochondria, it could be playing a role in A β processing at this site, suggesting that CyD may be important for the development of AD in humans. Although no other isoforms have been tested, CyD might have a unique effect on A β processing, possibly because it is the only cyclophilin isoform known to localize to the mitochondria. It is found that CyD overexpression in HEK cells could increase caspase-3 activity and stabilize PS1 NTFs suggesting it could directly modify PS1 activity. Furthermore, compounds that can bind and inhibit CyD activity, strongly inhibit both A β secretion and C99 cleavage.

In particular, Sangliferhin A strongly inhibits A β at 3 μ M and is not toxic to the cells until 20 μ M. Sangliferhin A is known to bind and inhibit the PPIase activity of CyD without affecting the ability of CyD to bind to the adenine nucleotide translocator (ANT) and does not inhibit calcineurin activity like CsA (Samantha J. Clarke 2002). The steep dose response of Sangliferhin A might be explained in several ways. Sangliferhin A may inhibit pore opening only when a significant portion of CyD is bound, enough to disrupt its activity at the MPT (Clarke 2002). Since the ANT exists as a dimer, it is likely CyD has to bind ANT with more than one molecule to induce a conformational change. Since Sangliferhin A has to bind multiple CyD molecules to inhibit its activity on the MPT complex, this would explain the rapid

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inhibition of A β secretion at the 3 μ M threshold. Once this threshold is reached the MPT complex can be completely inhibited and prevent pore opening. This threshold of MPT inhibition also seems to be an important regulator of A β processing and/or secretion. Alternatively, Sangliferhin A could have multiple targets other than or addition to CyD. Whether CyD is regulating the MPT complex directly through its PPIase activity or by a conformational change in the MPT, is not yet clear. In any case, the data presented here and by others are consistent with CyD and the MPT pore playing a key role in A β processing.

Although these compounds could dramatically decrease A β secretion and inhibit C99 cleavage, they also inhibited Notch cleavage. This was not surprising considering the data suggested CyD and CPZ overexpression affected GACE activity. This data is the first indication that immunophilin compounds can inhibit GACE activity and defines a new pathway that can influence A β and Notch processing in the cell.

Interestingly, FK506 treatment dramatically increases A β 42 secretion in a dose dependant fashion. FK506 is known to bind immunophilin proteins but not to CyD. The specific increase in A β 42 suggests that FK506 target proteins might be regulating A β 42 metabolism or production. Both FK506 and CsA can inhibit calcineurin but they have differential affects on A β secretion, thus calcineurin is probably not affecting A β levels. This unique affect of FK506 further supports the immunophilin pathway regulates A β processing.

Example 2

C99 and Notch cleavage

A HEK 293 stable cell line is generated using a modified C99 or Notch transmembrane sequence with a signal peptide, TGN retention sequence, and a GAL4-NLS-VP16 sequence inserted at Q56/Y57 as described in Maltrese, Wilson, et al. 2001. The cells also stably express a 5xGAL4RE-luciferase reporter construct (RD-2002-01437 and RD-2001-02419). These cells measure GACE activity when the C99 transgene is cleaved which activates the Gal4 luciferase reporter. These HEK stable cells are treated with cyclosporin A, Sangliferhin A, FK506 or N-methyl-4-valine-cyclosporin and the IC₅₀ for C99 cleavage are determined. GALVP alone may be as the negative control. The GALVP IC₅₀ for cyclosporin A, N-methyl-4-valine-cyclosporin Sangliferhin A, and FK506 is 6.9, 9.9, >20, and >20 μ M, respectively, indicating a clear window between inhibition of C99 cleavage and cell viability, since these compounds are not toxic until > 40 μ M. However, cyclosporin A, N-methyl-4-valine-cyclosporin Sangliferhin A all inhibit C99 cleavage at 0.71, 0.87, and 0.85 μ M, and inhibit

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Notch cleavage at 1.7, 2.7 and 3.2 μ M, respectively. FK506 has no effect on C99 or Notch cleavage. Therefore ligands that can bind CyD strongly inhibit C99 and Notch cleavage suggesting GACE activity is being inhibited.

Example 3

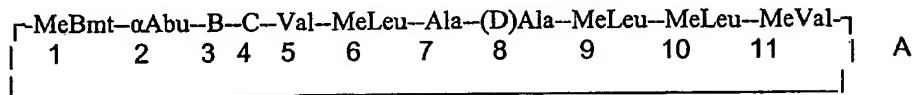
Caspase-3 activation

Recent studies suggest that caspase-3 activation can cause an increase in A β secretion and stabilize the proteins in the GACE complex (Tesco, Koh et al. 2003). Since CyD and CPZ both increase A β secretion and stabilize PS1 NTFs, the levels of caspase-3 activation are analyzed in CyD and CPZ overexpressing cells. HEK 293 cells are transiently transfected with CyD and CPZ along with APPwt for 24 hours, saponin permeabilized, fixed, and probed with a FITC conjugated monoclonal antibody to active caspase-3. Negative control cells are transfected with an empty vector and APPwt and positive control cells are treated with 1 μ M staurosporine for 6 hours prior to analysis. In cells expressing CyD, 52% of the cells tested positive for active caspase-3, while in CPZ expressing cells, 48% of the cells are positive for caspase-3. These results indicate that the overexpression of these proteins induce the activation of caspase-3 suggesting this is a possible mechanism by which PS1 NTFs are stabilized and A β secretion is increased.

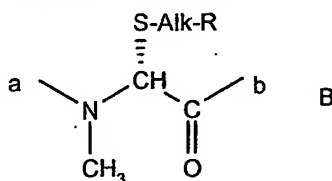
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CLAIMS

1. Use of a nonimmunosuppressive, cyclophilin-binding cyclosporin in the manufacture of a medicament for treating or preventing pathological conditions associated with A β production and/or secretion such as Alzheimer's Disease, Parkinson's Disease, tauopathies, prion diseases, frontotemporal dementia, striatonigral degeneration, Lewy body dementia, Huntington's disease, Pick's disease, amyloidosis, and other neurodegenerative disorders associated with excess A β production.
2. A method for the treatment or the prevention of pathological conditions associated with A β production and/or secretion, comprising administering to said patient an effective amount of a nonimmunosuppressive, cyclophilin-binding cyclosporin.
3. A use according to claim 1 or a method according to claim 2 in which the nonimmunosuppressive, cyclophilin-binding cyclosporin is a compound of Formula A



wherein B is an amino acid residue of formula B



wherein a denotes the bond to the α Abu residue in position 2;

b denotes the bond to the residue C in the 4 position;

Alk represents straight or branched chain alkylene containing from 2 to 6 carbon atoms or cycloalkylene containing from 3 to 6 carbon atoms, and

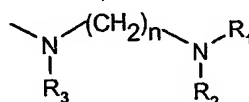
R represents

a carboxy or alkyloxycarbonyl radical;

a radical -NR₁R₂ in which R₁ and R₂ are the same or different and represent hydrogen, alkyl, C₂₋₄ alkenyl, C₃₋₆cycloalkyl, phenyl (optionally substituted by

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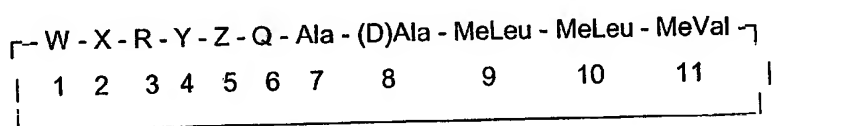
halogen, alkoxy, alkoxycarbonyl, amino, alkylamino or dialkylamino) or a benzyl or saturated or unsaturated heterocyclyl radical containing 5 or 6 ring atoms and 1 to 3 heteroatoms, or in which R₁ and R₂ form together with the nitrogen atom to which they are attached a saturated or unsaturated heterocycle containing 4 to 6 ring atoms and optionally containing a further heteroatom selected from nitrogen, oxygen or sulphur and optionally substituted by alkyl, phenyl or benzyl;
a radical of formula



wherein R₁ and R₂ are as defined above, R₃ represents hydrogen or an alkyl radical and n is a whole number from 2 to 4,
and wherein alkyl denotes straight or branched chain alkyl containing from 1 to 4 carbon atoms;

C is MeLeu or or 4-hydroxy-MeLeu; and the pharmaceutically acceptable salts thereof.

4. A use according to claim 1 or a method according to claim 2 in which the nonimmunosuppressive, cyclophilin-binding cyclosporin is a compound of Formula I:



in which W is MeBmt, dihydro-MeBmt or 8'-hydroxy-MeBmt;

X is αAbu, Val, Thr, Nva or O-methyl threonine (MeOThr);

R is Sar or (D)-MeAla;

Y is MeLeu, γ-hydroxy-MeLeu, Melle, MeVal, MeThr, MeAla, Me Tyr, MeTyr(O-PO(OH)₂), Mealle or MeaThr, or Pro;

Z is Val, Leu, N-Alk-Val or N-Alk-Leu,

wherein Alk represents Me or Me substituted by

vinyl optionally substituted by

phenyl, or an N S or O heteroaryl containing 6 ring members, or

phenyl optionally substituted by

halogen; and

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Q is MeLeu, γ -hydroxy-MeLeu or MeAla.

5. A use according to claim 1 or a method according to claim 2 in which the nonimmunosuppressive, cyclophilin-binding cyclosporin is a compound selected from the group comprising:
- a) [dihydro-MeBmt]¹-[γ -hydroxy-MeLeu]⁴-Ciclosporin;
 - b) [MeVal]⁴-Ciclosporin;
 - c) [Melle]⁴-Ciclosporin;
 - d) [MeThr]⁴-Ciclosporin;
 - e) [γ -hydroxy-MeLeu]⁴-Ciclosporin;
 - f) [Nva]²-[γ -hydroxy-MeLeu]⁴-Ciclosporin;
 - g) [γ -hydroxy-MeLeu]⁴-[γ -hydroxy-MeLeu]⁶-Ciclosporin;
 - h) [MeVal]⁵-Ciclosporin;
 - i) [MeOThr]²-[(D)MeAla]³-[MeVal]⁵-Ciclosporin, or
 - j) [8'-hydroxy-MeBmt]¹-Ciclosporin.
 - m) [N-Benzyl-Val]⁵-Ciclosporin,
 - n) [N-5-Fluoro-Benzyl-Val]⁵-Ciclosporin,
 - o) [N-Allyl-Val]⁵-Ciclosporin,
 - p) [N-3-Phenyl-Allyl-Val]⁵-Ciclosporin,
 - q) [Pro]⁴-Ciclosporin, or
 - r) [γ -hydroxy-MeLeu]⁹-Ciclosporin.
6. A use according to claim 1 or a method according to claim 2 in which the nonimmunosuppressive, cyclophilin-binding cyclosporin is [MeVal]⁴-Ciclosporin.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP2005/007556

A. CLASSIFICATION OF SUBJECT MATTER

A61K38/13 C07K7/64 A61P25/00 A61P25/28 A61P25/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, EMBASE, BIOSIS, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	WO 97/18828 A (GUILFORD PHARMACEUTICALS INC; JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDIC) 29 May 1997 (1997-05-29) abstract; claims 39,53,54,57,58; example 4; tables 1,2	1-6
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☒ Further documents are listed in the continuation of box C

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

8 December 2005

Date of mailing of the international search report

20/12/2005

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Vandenbogaerde, A

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2005/007556

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